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Preface

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The Murine Allantois

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I. Introduction

The focus of this chapter is the development of the mouse allantois. The murine allantois is the future umbilical component of the chorioallantoic placenta. Its primary function is to fuse with the chorion and vascularize, thereby serving as the vital connection between mother and fetus for the interchange of nutrients, gases, and metabolic wastes during much of mammalian gestation. Whereas much effort has been devoted to investigating the role of the mature placenta, studies of the development of its component structures, the chorion and the allantois, have been minimal.

Until now, there have been no reviews on the murine allantois, probably because few studies have focused on it. This may account for the fact that even where there has been modest opportunity to increase understanding of allantoic growth and morphogenesis through gene expression studies or examination of transgenic mice mutant in vasculogenesis, the allantois has been treated superficially or ignored altogether. There has been little context for gene expression

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and transgenesis, the significance of which can only be appreciated in any developing system if the fate and state of differentiation of its component cells are known.

The aim of this review is to synthesize what is known about early development of the murine allantois. Topics to be addressed include the embryonic origin of the allantois, how the allantois acquires its unusual shape, and the mechanism of chorioallantoic fusion, which establishes the early placenta. A model is proposed for how the murine allantois may employ its unique morphology to undergo vasculogenesis. Whether the allantois plays a role in the formation of the future germ line will also be discussed. The chapter will conclude with the author's long-term view that the allantois, with its direct connection to fetal circulation, may be manipulated to deliver therapeutic factors to certain developmentally compromised fetuses.

II. Fetal Membranes: Overview

In the development of any vertebrate embryo, only part of the egg's cleavage cell mass will form the actual embryo; the other part will elaborate the extraembryonic structures, referred to especially in mammals as fetal membranes. The set of fetal membranes comprises the yolk sac, amnion, chorion, and allantois. Fetal membranes eventually are shed at birth by natural methods and discarded.

The yolk sac is the most variable fetal membrane of animals (Hamilton *et al.*, 1947). The mammalian yolk sac, although not functional in the sense of storing yolk, is so similar in many details to the reptilian yolk sac as to be homologous (Snell and Stevens, 1966). In mammals, the yolk sac plays a significant role in the uptake and transfer of nutrients to the embryo by way of the exocoelomic cavity (Wislocki *et al.*, 1946). Combined with the observation that the yolk sac vascularizes very early and engages in erythropoiesis, the yolk sac functionally is an organ of nutrition and gas exchange and is considered a proper placenta. In most marsupials it is the only placenta (Pijnenborg *et al.*, 1981). The yolk sac is also thought to seed the fetus with hematopoietic stem cells [reviewed in Morrison *et al.* (1995)]. In humans, yolk sac function diminishes very early, but the yolk sac itself persists through the remainder of gestation as a rudimentary organ, a constituent of the mature umbilical cord (Boe, 1951; see the following). In contrast, in small rodents, the yolk sac is an important nutritive organ during the full period of gestation (Everett, 1935), although the chorioallantoic placenta supersedes it in physiological importance.

The chorioallantoic placenta is formed by the union of two initially well-separated structures, the chorion and the allantois. The most significant feature of the allantois is that it vascularizes. Development of the allantois varies between animal species (Panigel, 1993). The allantois was evolved by reptiles and birds as a temporary sac for the storage of urinary waste [Arey, 1965; a comparative

Karen M. Downs

anatomy of the allantois has been reviewed in Steven and Morriss (1975)]. In these animals, fusion of the outer wall of the allantoic sac with the chorion produces a structure in direct contact with the porous shell. The allantois develops a vasculature, and oxygenation takes place in the vascularized wall of the sac. The allantoic sac continues to serve as a reservoir for kidney excreta, and part of the allantoic wall assists in the absorption of egg albumen.

Monotremes [egg-laying mammals such as the echidna (or spiny anteater) and platypus] possess an allantoic sac whose function is comparable to that of reptiles and birds. In most marsupials, the allantois fails to grow far enough to reach the chorion, which is found in most marsupials and consists of ectoderm and avascular somatic mesoderm. Nitrogenous waste therefore is retained in the allantois until parturition (Morriss, 1975). Only one marsupial (*Perameles* or bandicoot) possesses a vascular chorioallantoic unit, which is apposed to the uterine mucosa (Panigel, 1993). In this species, the chorioallantois is important in hematrophic nutrition of the fetus (Morriss, 1975).

In humans, the allantois consists of endoderm and mesoderm and is the scaffolding upon which the mature umbilical cord is formed. The mature human umbilical cord is a composite structure, formed when the allantois-derived connecting stalk and vitelline duct of the yolk sac are bound together by the expanding amnion (Larsen, 1993). An umbilical vein and two arteries develop within the connecting stalk and vascularize the chorionic disk. In mice, the allantois is wholly mesodermal (Snell and Stevens, 1966); allantoic mesoderm differentiates into a major artery and vein and surrounding connective tissue. This simple outcropping of the anteroposterior axis (see the following) becomes the umbilical component of the murine chorioallantoic placenta.

III. Development of the Exocoelomic Cavity

Development of the murine allantois is intimately associated with the development of the exocoelomic cavity, or exocoelom, which contains the yolk sac, amnion, chorion, and allantois, all of whose correct morphogenesis and function are essential for fetal health, survival, and development during the second half of gestation [Fig. 1; see Jolly and Férester-Tadié (1936) and Bonnevie (1950) for meticulously detailed classical papers on the development of the murine exocoelom]. The yolk sac is the first site of hematopoiesis [see Haar and Ackerman (1971) for a detailed morphological description of the early development of the yolk sac], and the amnion serves a protective function and permits the fetus to move freely, thus aiding in growth and development (Moore, 1982). The chorion and the allantois together comprise the major components of the placenta.

The exocoelom develops during gastrulation, the time during which the embryonic epiblast lays down the basic body plan of the fetus [for a classic review, see Beddington (1983); Lawson *et al.*, 1991; Lawson and Pedersen, 1992]. At the

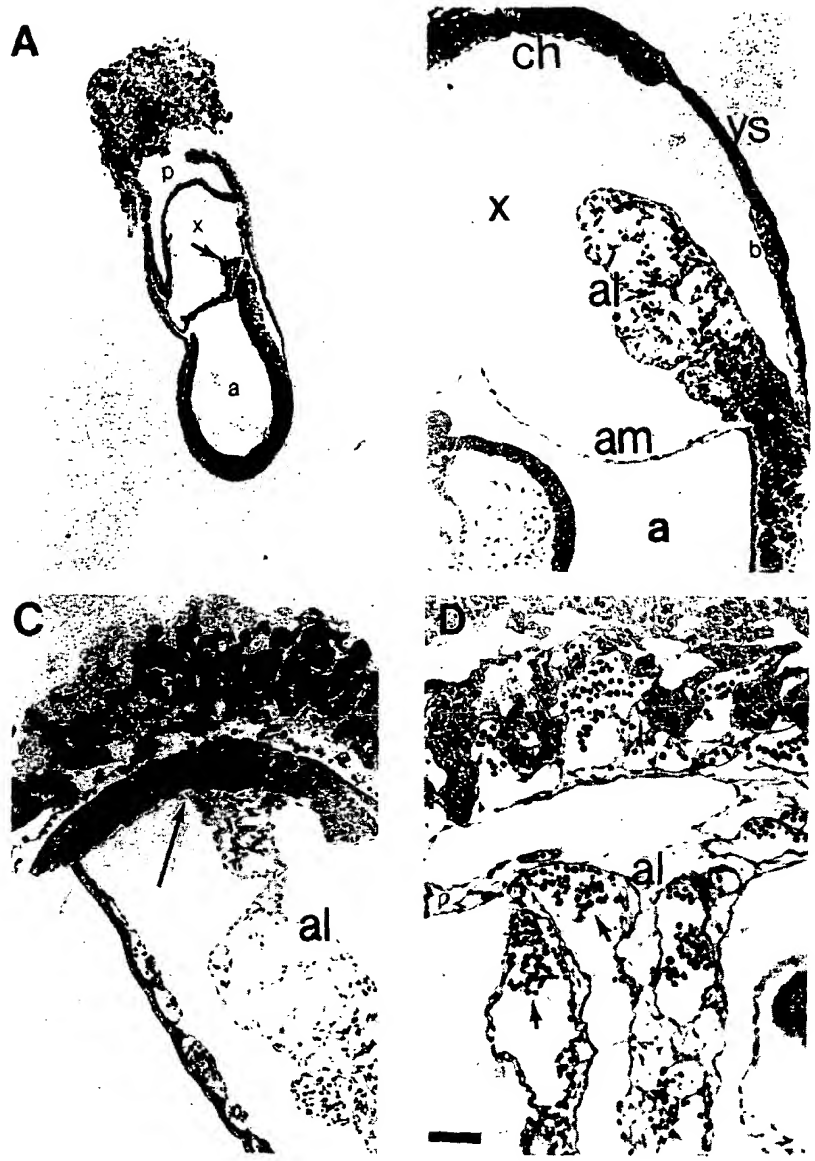
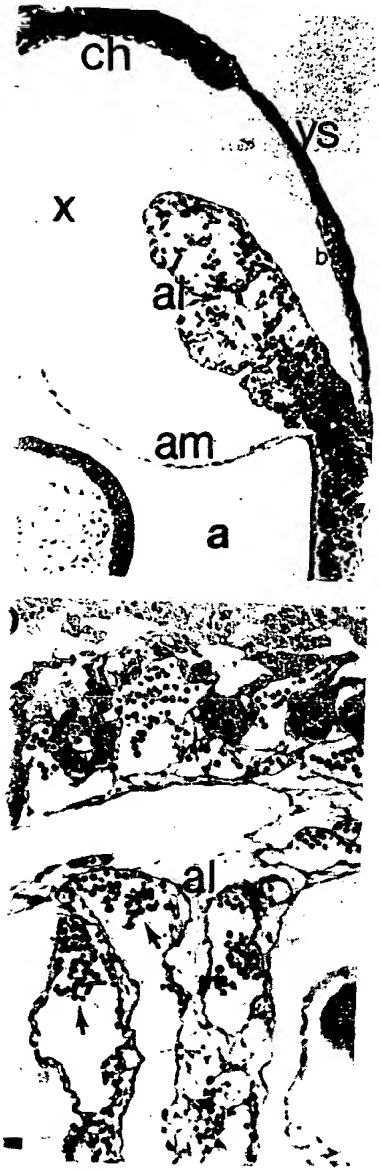


Fig. 1 Allantoic development. (A–D) Histological sections of PO conceptuses (Kelly and Rossant, 1976; Downs and Gardner, 1995) were prepared for visualization of the four phases of allantoic development. (A) Formation of the allantoic bud. Neural plate stage (7.25–7.5 dpc). The arrow indicates the allantoic bud. (B) Growth of the allantois. Early somite stage (8.0–8.25 dpc). (C) Chorioallantoic fusion (8.5 dpc), seven somite stage. The arrow points to the chorioallantoic fusion junction. (D) Vascularization of the allantois. Conceptus (9.5 dpc) showing overt vascularization of



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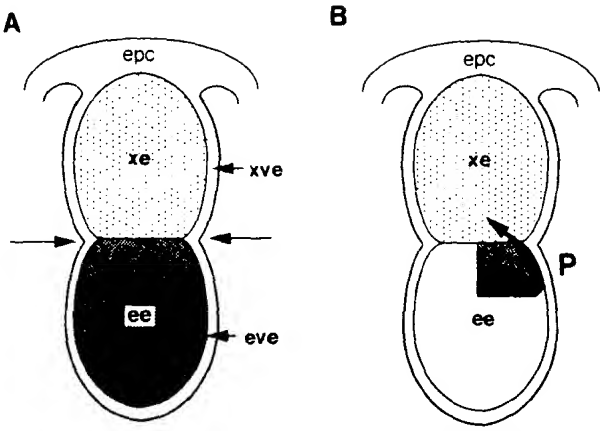

Fig. 2 Schematic diagram of the mouse egg cylinder just before and after the onset of gastrulation. The mouse egg cylinder is divided into two major regions just before (A) and after (B) the onset of gastrulation. In A, the unlabeled arrows separate extraembryonic ectoderm (xe) from embryonic ectoderm (epiblast or ee). Both the extraembryonic and embryonic ectoderms are surrounded by a single layer of visceral endoderm: xve, extraembryonic visceral endoderm; eve, embryonic visceral endoderm. Other abbreviations: epc, ectoplacental cone. In B, gastrulation has begun as indicated by formation of the primitive streak (solid black area in the embryonic portion of the egg cylinder) adjacent to embryonic visceral endoderm. The appearance of the streak indicates the posterior (P) region of the future fetus. The proximal epiblast indicated just below the embryonic–extraembryonic junction is ingressing (horizontal arrow) into the posterior primitive streak and emerging from the latter (arrow going into the extraembryonic region) as extraembryonic mesoderm.

onset of gastrulation, approximately 6.5 days postcoitum (dpc), the conceptus consists of two major areas, the extraembryonic and embryonic regions [Fig. 2A; described in Snell and Stevens (1966); Hogan *et al.*, 1994; also, a morphological staging system of gastrulation is found in Downs and Davies (1993)]. The extraembryonic region encompasses multiple tissue types, but the two important to the formation of the exocoelom are the ectoderm of the future chorion and the endoderm of the future yolk sac. The embryonic region primarily consists of a pluripotent epithelium called the "primitive ectoderm" or "epiblast," which is surrounded by a layer of (extraembryonic) visceral endoderm (Fig. 2A).

the umbilical component of the chorioallantoic placenta. Arrowheads indicate red blood cells. Abbreviations: a, amniotic cavity; al, allantois; am, amnion; b, yolk sac blood island; ch, chorion; p, ectoplacental cavity; ys, yolk sac; x, exocoelomic cavity. Scale bar: 100 μm (A, B); 50 μm (C); 68 μm (D). Methods: (A and D) Bouin's-fixed, paraffin-embedded conceptuses were sectioned at 7 μm and stained in hematoxylin and eosin. (B and C) Glutaraldehyde-fixed, araldite-embedded conceptuses were sectioned at 1 μm and stained in toluidine blue.

The key feature of gastrulation is diversification of the epiblast. The epiblast alone is responsible for laying down all of the tissues of the definitive mouse embryo (Gardner and Rossant, 1979; Gardner *et al.*, 1985). In addition, the epiblast forms the extraembryonic mesoderm and all of the amnion. The general sequence of epiblast diversification is that it lays down first the primitive streak (Fig. 2B; Bonnevie, 1950), second the extraembryonic mesoderm (Lawson *et al.*, 1991), and third the three embryonic germ layers, ectoderm, mesoderm, and endoderm, as well as the ectoderm of the amnion (Lawson *et al.*, 1991; Lawson and Pedersen, 1992; Lawson and Hage, 1994). The germ layers subsequently will interact according to their level relative to the streak and set off the cascade of events necessary for the formation of all of the definitive organs.

Although the details are far from clear, mesoderm is formed by the ingression of epiblast into the primitive streak (Beddington, 1981, 1982; Copp *et al.*, 1986; Tam and Beddington, 1987; Lawson *et al.*, 1991; Lawson and Pedersen, 1992). As gastrulation proceeds, the primitive streak lengthens anteriorly, and extraembryonic, lateral plate, paraxial, and axial mesoderms are specified by the ingression of epiblast cells into progressively more anterior levels of the streak (Tam and Beddington, 1987).

Germane to this review is extraembryonic mesoderm. Extraembryonic mesoderm is formed as a result of ingression into the posterior primitive streak by proximal embryonic epiblast, which is that epiblast situated just below the embryonic–extraembryonic junction of the egg cylinder [Fig. 2B; Lawson *et al.*, 1991; reviewed in Boucher and Pedersen (1996)]. Extraembryonic mesodermal cells emerge from the streak as a bulge overlying and continuous with it. Anteriorly, a smaller bulge of extraembryonic mesoderm forms. Cavitation within these masses of extraembryonic mesoderm results in the formation of two amniotic folds that fuse, thereby forming the exocoelom. This results in the partitioning of the egg cylinder into distinct regions dominated by three cavities: amniotic, exocoelomic, and ectoplacental (Beddington, 1983).

In the exocoelom, extraembryonic mesoderm complexes with extraembryonic ectoderm to form the chorion and with extraembryonic endoderm to form the yolk sac. Eventually the amnion will appear; it consists of ectoderm and mesoderm and will comprise the floor of the exocoelom. The allantois will form at the angle between the yolk sac and amnion. It consists wholly of extraembryonic mesoderm.

The sequence of events by which extraembryonic mesoderm contributes to the components of the exocoelom has not been described. However, results of fate mapping of the epiblast and the posterior primitive streak at the onset of gastrulation and through the three-somite stage (approximately 8.25 dpc) have tentatively suggested that the extraembryonic mesoderm may contribute to exocoelomic structures in three successive waves. The first wave occurs at the onset of gastrulation; the first extraembryonic mesoderm contributes to all four components of the exocoelom. This is quite clear, because descendants of labeled proximal

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epiblast cells were found in the mesoderm of the chorion, yolk sac, amnion, and allantois at the experimental endpoint of fate-mapping experiments (Lawson *et al.*, 1991). In the second wave (mid-to-late streak stages; Downs and Davies, 1993; K. Downs, preliminary observations), extraembryonic mesoderm arising from the posterior streak may contribute only to the allantois and amnion. In the third wave (neural plate and headfold stages; Beddington, 1982; Copp *et al.*, 1986; Tam and Beddington, 1987), the majority of extraembryonic mesodermal cells emerging from the posterior streak were found largely in the allantois, although a few grafted cells were also identified in yolk sac mesothelium (Tam and Beddington, 1987). Colonization of the yolk sac at this time may be explained by contamination with the donor graft during transplantation (Downs and Harmann, 1997).

Once the addition of extraembryonic mesoderm to the allantois ceases by three somite pairs (Tam and Beddington, 1987; Downs and Gardner, 1995), formation of the exocoelom is complete. This putative sequence of mesoderm deposition into components of the exocoelomic cavity may be critical for elucidating the mode of action of the *Brachyury* mutation of the allantois (discussed in Section V.B).

IV. Development of the Allantois

Little is known about allantoic development, but allantoic morphology and behavior suggest a convenient quadraphasic sequence of events: (A) formation of the allantoic bud (Fig. 1A), (B) allantoic growth (Fig. 1B), (C) chorioallantoic fusion (Fig. 1C), and (D) overt vascularization of the allantois (Fig. 1D).

A. Formation of the Allantoic Bud

The timing of appearance of the allantoic bud has been reported in two mouse strains. In the first, the allantoic bud is visible shortly after cavitation of extraembryonic mesoderm [late streak stage, approximately 7.0 dpc, F_2 generation of (C57BL/6 × CBA) hybrid intercrosses; Kaufman, 1992]. In the second strain, the bud is apparent after fusion of the proamniotic folds and formation of the amnion (Fig. 1A; early neural plate stage, approximately 7.25 dpc, closed-bred Swiss-derived albino; Beddington, 1983; Downs and Davies, 1993). Despite these slight strain-dependent differences in timing of appearance, the cellular composition of the allantois is not thought to differ between strains.

The allantoic bud lies suprajacent to and is continuous with the posterior primitive streak. The outer cells of the bud will differentiate into a layer of mesothelium that surrounds an inner core of extraembryonic mesoderm. In the rat, the apical surface of the mesothelial layer contains no modifications, but the

basal membrane of these cells bears small processes that project into the subjacent extracellular space (Ellington, 1985).

How the allantoic bud is formed is not known, but one possibility is that it does so through a decline in the rate of proliferation of the first wave of extraembryonic mesoderm once it has become complexed with extraembryonic ectoderm and endoderm. This would result in the production of excess mesoderm by continued ingression of proximal epiblast into the posterior primitive streak. That surplus extraembryonic mesoderm becomes the allantois.

B. Growth of the Allantois

The allantois appears to enlarge by the addition of mesoderm from the posterior streak until the three somite stage (Tam and Beddington, 1987). After this time, a combination of mitosis and distal cavitation may be responsible for its continued growth in the exocoelom (Ellington, 1985).

During growth, the allantois is cylindrical, approximately 0.5 mm long, 0.18 mm wide at the base, and tapering to 0.08 mm wide near the tip (Tamarin and Boyde, 1976). Nevertheless, some variation is observed between conceptuses at similar developmental stages (Downs and Gardner, 1995). Also during growth, the base of the allantois becomes hollowed out, and the inner surface consists of a discontinuous pavement of cells containing multiple processes (Tamarin and Boyde, 1976). The significance of these morphological features is not known.

The outer mesothelium is most conspicuous during the allantoic growth phase; it is continuous with the mesodermal layer of the yolk sac, chorion, and amnion. In the rat, the mesothelial layer acquires desmosomes and exhibits bulbous protrusions whose significance is unclear. Distally, these protrusions may be involved in chorioallantoic fusion (Ellington, 1987), and proximally, they resemble the blood islands of the yolk sac (Ellington, 1985; Tamarin and Boyde, 1976).

How the allantoic mesothelial cells become morphologically distinct from underlying core cells is unknown, but it may be by virtue of their external position, similar to the positional differentiation of trophectoderm cells in the morula (Tarkowski and Wroblewska, 1967; Barlow *et al.*, 1972). Electron micrograph studies do not report a basement membrane subjacent to the mesothelial cells (Ellington, 1985), which suggests that sustained growth of the mesothelium during the growth phase may be due to intercalation of cells from the allantoic core rather than self-propagation of the outer mesothelial cell layer.

During growth, distal inner core allantoic mesoderm becomes loosely organized with the distance between cells increasing, with some cell contacts but no specialized junctions (Ellington, 1985). Cells in the proximal allantois remain densely packed and always continuous with the posterior streak. The line of demarcation between the base of the allantois and the subjacent primitive streak

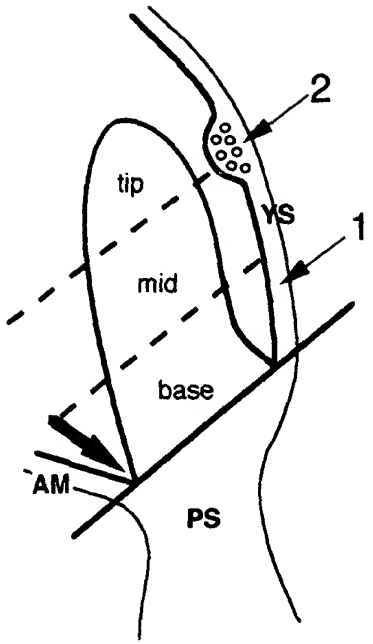

Fig. 3 Schematic diagram of the headfold-stage allantois. The arrow indicates the insertion of the amnion (AM) used to delineate the base of the allantois from the subjacent primitive streak (PS; Oździenski, 1967). The bold line surrounding the allantois, which is continuous with the yolk sac, amnion, and chorion (not shown), represents the mesodermal lining of the exocoelom. The headfold-stage allantois was subdivided (dashed lines) into three major regions, base, midregion, and tip, for studies of allantoic potency (Downs and Harmann, 1997). Other abbreviations: YS, yolk sac.

is typically taken as the level of the amnionic extension from the base of the allantois (Oździenski, 1967; Fig. 3).

A few gene expression studies have revealed that some biochemical differences may be found between the base and the distal two-thirds of the allantois. For example, *Brachyury* is expressed in the base of the allantois in the headfold and early somite stages (Clements *et al.*, 1996), whereas CD44, the major receptor of hyaluronan (Aruffo *et al.*, 1990), appears everywhere but in the core of the base at this time (Fig. 4). *VCAM1*, required for chorioallantoic fusion (Yang *et al.*, 1995), is expressed in the distal two-thirds of the allantois as early as what appears to be the four-somite stage (Kwee *et al.*, 1995). There is also a small population of alkaline phosphatase-positive cells in the base of the allantois, which may contribute to somatic lineages (e.g., the mature allantois), the future germ line, or both (Oździenski, 1967; discussed in Section V.C).

Addition of extraembryonic mesoderm to the allantois ceases by three somite pairs. This was demonstrated in experiments where the posterior primitive streak

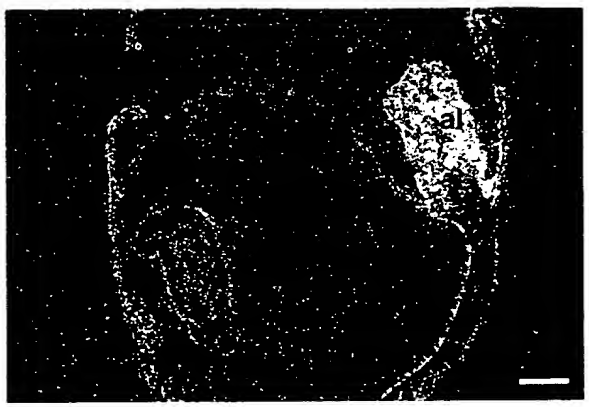

Fig. 4 Expression of CD44 mRNA in the allantois of an early somite stage conceptus. Histological section showing mRNA hybridization *in situ* of an early somite-stage conceptus to CD44 cDNA. Arrow indicates the allantois. Note that the base of the allantois exhibits relatively little, if any, positive signal for CD44, whereas the distal allantois is strongly positive. Scale bar: 100 μm. Methods: Hybridization was originally described in Downs (1992). A CD44 cDNA probe (gift of Dr. E. Shtivelman, Systemix, Palo Alto, CA) was restriction-digested with appropriate restriction enzymes, gel-purified, and random-primed with [^{33}P]-dATP to specific activity of 10^8–10^9 cpm following the instructions in the Boehringer-Mannheim Random-Primed DNA Labelling Kit ([^{33}P]-dATP, sp. act. 3000 Ci/mmol, NEN Dupont, UK). Paraffin sections (6 μm) of mouse embryos in early somite stages were prepared as described by Wilkinson and Green (1990) with the following exceptions: (1) Histoclear (National Diagnostics, Manville, NJ) was used to clear the tissue instead of toluene (less toxicity) before embedding. (2) 0.85% NaCl was eliminated from the dehydration ethanols and the several rinse steps. Sections were hybridized for 3 days at 42°C under clean cover slips at a probe concentration of 0.5 ng/μl and washed as previously described (Downs *et al.*, 1989). The slides were then dehydrated as described, coated once with NTB-2 emulsion (diluted 5 parts emulsion to 7 parts 1% glycerol), and left to dry vertically in air for 1 hr. Slides were then exposed for 8–20 days in sealed, light-tight boxes in the presence of desiccant at 4°C and brought to room temperature for development. The tissue was stained in Mayer's hematoxylin alone or counterstained in eosin (and cover-slipped in DPX mounting medium). All photographs were taken on a Zeiss Axioplan and processed for identical exposure times. The control probe was α-fetoprotein and was applied to alternating serial sections as previously described (Downs *et al.*, 1989).

was orthotopically transplanted at the late neural plate–early headfold stage and the fate of donor cells followed. No contribution of grafted cells was observed in the allantois beyond three somite pairs (Tam and Beddington, 1987). Also, in experiments where the allantois had been removed, a new allantois regenerated and fused with the chorion before, but not after, this developmental time (Downs and Gardner, 1995).

The rate of mitosis within the rodent allantois declines steadily until fusion (Ellington, 1985). There does not appear to be a proliferating center within the allantois itself, although the fine region-specific mapping required to find such a center has not been carried out.

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C. Chorioallantoic Fusion

By the 6–7 somite stage (approximately 8.25 dpc), the allantois meets the chorion and attaches to it (Theiler, 1989; Downs and Gardner, 1995). This first step in placental morphogenesis is mediated by the mesothelial surfaces of the allantois and chorion (Ellington, 1985; Downs and Gardner, 1995). Fusion occurs shortly thereafter. The vital link between fetus and mother has thus been forged.

The events that take place during fusion are far from clear. In most rat embryos examined, the tip of the allantois settles into a depression in the chorion (Ellington, 1985). This has also been described in less detail in the mouse (Tamarin and Boyde, 1976). However, in a few rat embryos, the allantois first comes into contact with the posterior peripheral region of the chorion and then spreads along the surface of the chorion until contact and fusion are made in the central region of the chorion (Ellington, 1985). In the central chorionic depression, the cells are rounded up. This morphological feature may be of important functional significance, as chorioallantoic fusion has never been observed in areas of flattened chorionic mesoderm (Ellington, 1987).

What happens subsequent to chorioallantoic fusion is not clear. Attachment of the allantois to the chorion does not appear to be required for the formation of allantoic blood vessels or the infiltration of red blood cells into the allantois (Downs and Gardner, 1995). However, secretion of hepatocyte growth factor–scatter factor by the allantois subsequent to fusion with the chorion may stimulate the growth of trophoblastic cells in the labyrinth region of the chorionic disk (Uehara *et al.*, 1995).

D. Vasculogenesis in the Allantois

The mature murine umbilical cord consists of a single artery and vein. The allantois is not overtly vascularized until some time after chorioallantoic fusion, although it appears that allantoic vasculogenesis begins before this time (Yamaguchi *et al.*, 1993; Ellington, 1985; Downs and Harmann, 1997). Thus, angioblast formation and coalescence into rudimentary blood vessels do not depend upon interaction with the chorion (Jolly and Férester-Tadié, 1936; Downs and Gardner, 1995; Downs and Harmann, 1997).

By about 10 dpc in the rat (approximately four somite pairs in the mouse), rudiments of the allantoic vascular system appear as long, thin cytoplasmic processes joining allantoic cells together, forming small vesicles that later coalesce to form blood vessels (Ellington, 1985). Also during this time in the mouse, *flk*-1, a tyrosine kinase receptor whose ligand is vascular endothelial growth factor (VEGF), is expressed in individual cells of the allantois (Yamaguchi *et al.*, 1993). VEGF appears to be involved in many functions of endothelial cells, including proliferation and vascular permeability [reviewed in Mustonen and Alitalo (1995); Shibuya, 1995].

Studies designed to discover directly when angioblasts are present in the allantois were carried out by heterotopic transplantation. These experiments revealed that angioblasts are present in the allantois as early as the headfold stage (approximately 8.0 dpc; Downs and Harmann, 1997). The source of the angioblasts is likely to be the allantois itself, as cells from the yolk sac, all of which are red blood cells, do not infiltrate the allantois until possibly as late as the 10 somite stage. This is approximately 22 hr after transplantations at the headfold stage were carried out (K. Downs, S. Gifford, and M. Blahnik, preliminary data). A model for how angioblasts are formed in the allantois will be discussed in Section VI.A.

V. Unique Characteristics of the Allantois

A. The Mechanism of Chorioallantoic Fusion

The allantois grows into the exocoelomic cavity and attaches to and fuses with the chorion. Fusion is defined here as a union of tissue through mutual adhesion; there is no evidence or belief that actual cell fusion occurs. The mechanism of chorioallantoic fusion can be envisioned by one of three models: (1) directed growth of the allantois, where initial contact between the allantois and the chorion would be sufficient to stimulate the expression of appropriate cell adhesion molecules; (2) selective adhesion involving molecules specific to the allantois and chorion that might be expressed constitutively on both of these structures during their growth phase; or (3) selective adhesion involving expression of the requisite molecules but that might be developmentally regulated during maturation of one or the other structure.

The models were then tested (Downs and Gardner, 1995). Experiments were carried out in which distal halves of donor allantoises were placed individually into the exocoelomic cavity of similar-stage hosts whose own allantois had been removed (Fig. 5). This manipulation allowed the donor allantois the freedom to select the exocoelomic surface to which it most preferred to adhere and revealed how early fusion could occur if the opportunity presented itself.

In *ex vivo* and cultured control conceptuses, chorioallantoic fusion began at about four somite pairs and reached a maximum by six somite pairs (Downs and Gardner, 1995). Maximal fusion by six somite pairs was in agreement with a previous report (Theiler, 1989). Thus, the use of whole embryo culture to study chorioallantoic fusion was validated.

In the operated conceptuses, most donor allantoic tips attached solely to the chorion, revealing that fusion was specific. Chorioallantoic attachment occurred first between the mesothelial surfaces and was followed by the intimate juxtaposition of allantoic and chorionic cells. Further, attachment occurred at the appropriate developmental stage, beginning at four somite pairs and reaching a

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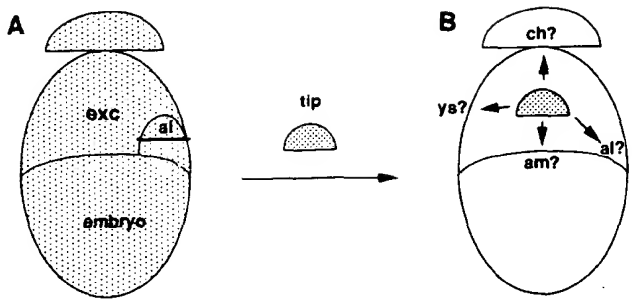

Fig. 5 *Schematic diagram of the microsurgical technique used to discover the mechanism of chorioallantoic fusion.* (A) The allantoic tip of a [^{3}H]methylthymidine-labeled conceptus was introduced into (B) the exocoelomic cavity from which the host allantois had been removed. The operated conceptus was then cultured for various times and scored for fusion of the tip to component tissues of the exocoelom. This microsurgical technique has been described in Downs and Gardner (1995). Abbreviations: al, allantois; am, amnion; ch, chorion; exc, exocoelom; ys, yolk sac.

maximum rate by six somites (Downs and Gardner, 1995). Thus, even in isolation and without continuity with the basal portion, donor allantoic tips behaved as they would *in situ*, fusing at the appropriate developmental stage. It appeared that a clock had been set to go off in the tip at the correct developmental time prior to loss of its connection with the base.

These experiments resolved the three possible models of chorioallantoic fusion. Although the donor allantoic tip was often found wedged in between the amnion and chorion before four somite pairs were attained by the host, no enduring attachment of the allantois to these structures had occurred, demonstrating that cell–cell interactions between the allantois and chorion do not trigger expression of the required cell adhesion molecules. Thus, the model of directed growth of the allantois as the driving mechanism of chorioallantoic fusion was eliminated. Moreover, expression of the requisite adhesion molecules was not constitutive, or the allantois and chorion would have fused prematurely because they were often positioned to do so. This observation eliminated the second model. Rather, chorioallantoic adhesion was selective and involved gradual acquisition of the specific adhesion molecules, beginning at about four somite pairs and increasing over the next several hours. This was borne out in heterochronic transplantations, where the allantois was either younger or older than the host chorion. Fusion was dependent upon the developmental maturity of the allantois, whereas the chorion was always receptive to a mature allantois.

The host embryos, in which the allantois had been removed, revealed two important properties. Where the allantois did not regenerate a significant new allantois, fetuses nonetheless were able to continue development over the next 34 hr, acquiring the same morphological landmarks as the unoperated controls (Downs and Gardner, 1995). In addition, axial rotation of the deleted mouse

embryos did not appear to be affected in the absence of an allantois, although one report has suggested that chorioallantoic fusion may be required for determining the sidedness of tail rotation in the rat (Fujinaga and Baden, 1993).

Thus, at least in the mouse, a mature chorioallantoic placenta does not appear to be essential for gross fetal development during the headfold stage, through about 18 somite pairs. The presence of the rodent yolk sac, which serves as a functional placenta early in gestation, makes the living conceptus amenable to manipulation outside of the mother during gastrulation [reviewed in Freeman (1990)].

1. Genetic Control of Chorioallantoic Fusion

Several gene deficiencies appear to affect the ability of the murine allantois to engage in fusion with the chorion. These deficiencies may involve defective proliferation of the allantois, preventing it from growing far enough to reach the chorion to fuse with it, for example, *LIM1* (Shawlot and Behringer, 1995), or they may affect allantoic morphogenesis, as in *Brachyury* mutants (Beddington *et al.*, 1992; discussed in Section V.B). The gene products of vascular cell adhesion molecule (*VCAM1*) and its receptor, the α4 subunit of integrin (α4-integrin) have been implicated as principal players in chorioallantoic fusion (Gurtner *et al.*, 1995; Kwee *et al.*, 1995; Yang *et al.*, 1995). Transgenic mice deficient in these protein products failed to exhibit chorioallantoic fusion and died at approximately 11.0–11.5 dpc.

Integrins are involved in many cellular processes, including cell proliferation, cell differentiation, cell migration, cytoskeletal organization, and cell polarization (Yang *et al.*, 1995). Integrins are heterodimers that contain α- and β-subunits. α4-integrin is expressed on hematopoietic stem cells and several types of leukocytes. *VCAM1* is expressed on the endothelium of blood vessels during inflammation and mediates leukocyte migration from blood into tissues. This receptor–ligand complex also appears to play a role in myogenesis, as α4-integrin is expressed on primary myotubes and *VCAM1* is expressed on secondary myoblasts, both of which will fuse to form skeletal muscle (Rosen *et al.*, 1992).

During chorioallantoic development, *VCAM1* and α4-integrin are expressed in the allantois and chorion. *VCAM1* is expressed in the allantois as early as the four-somite stage (Kwee *et al.*, 1995), and its receptor, α4-integrin, is expressed constitutively on the chorion (Yang *et al.*, 1995; Kwee *et al.*, 1995). These expression data would appear to fulfill the predictions of the mechanism of acquired adhesion described in the previous section (Downs and Gardner, 1995). However, the earliest time at which *VCAM1* is expressed is not known, and it is not clear why *VCAM1* is expressed in the distal two-thirds of the allantois instead of on the mesothelium alone. It may be that *VCAM1* is also involved in vasculogenesis of the allantois, which, during the allantoic growth phase, appears

Karen M. Downs

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VCAM1 and α4-integrin may not be the only genes required for chorioallantoic fusion. When null alleles of these genes were created in transgenic mice, chorioallantoic fusion was not prevented in all cases. This lack of penetrance suggests that other genes are involved.

2. Allantoic Proliferation vs Allantoic Adhesion in Embryos Defective in Chorioallantoic Fusion

Without microsurgical intervention, it is impossible to know whether the inability of the allantois to fuse with the chorion in certain genetic mutants, such as *Brachyury* (Glueksohn-Schoenheimer, 1944), *Csk1* (Thomas *et al.*, 1995), or *LIM1* (Shawlot and Behringer, 1995) is the result of inadequate proliferation of the allantois or defective expression of adhesion molecules required for attachment and/or fusion with the chorion. The microsurgical technique described here to discover the mechanism of chorioallantoic fusion may be invaluable for such studies. For example, appropriately labeled mutant allantoises could be excised from donor embryos and introduced into the exocoelomic cavity of wild-type hosts, and the operated hosts could be cultured until the time of fusion and subsequently analyzed for adhesion to the chorion. These experiments would reveal whether the allantois is defective in adhesion to the chorion. In reciprocal experiments, wild-type allantoises could be placed into the exocoelomic cavity of mutant hosts to discover whether the absence of fusion is due to defective functioning of the chorion.

B. Allantoic Morphology

It is not known how the allantois acquires its unique morphology, but rodlike extension into the exocoelom does not appear to be an intrinsic property of the allantois. This is because removal of the allantois at the headfold and early somite stages followed by growth in roller culture results in the rapid loss of its fingerlike shape and transition into a spherical mass of cells (Fig. 6A; Downs and Harmann, 1997). The mass undergoes vasculogenesis only rarely (Downs and Harmann, 1997). From this, it is tempting to speculate that the characteristic shape of the allantois may be the result of its proximity to and wedging between the amnion and yolk sac, which together place restraints upon the allantois and mold it into a rod during the growth phase (Fig. 6B). Some morphological evidence that the allantois makes "traction" on the amnion during growth, utilizing what appear to be the bulbous projections on the mesothelial surface of the allantois, has been reported previously (Downs and Gardner, 1995). The adhe-

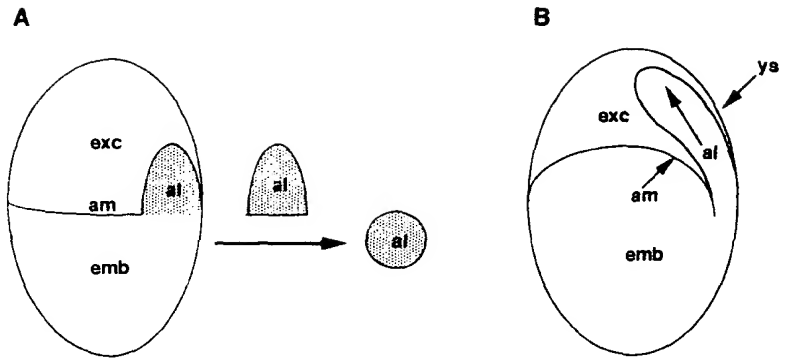


Fig. 6 Hypothetical mechanism for shaping the allantois. (A) Schematic diagram depicting the results of culture of the allantois in isolation (Downs and Harmann, 1997). Headfold-stage allantoises (stippled marks) were removed via yolk sac puncture and cultured for 24 hr, after which they assumed the shape of nearly perfect spheres. (B) Schematic diagram of the hypothetical mechanism proposed by the author to account for the shape of the allantois. The intact allantois is molded by contact with the yolk sac and amnion. During this time, the allantois may make transitory contacts with the amnion (Downs and Gardner, 1995). Abbreviations: emb, embryo; see Fig. 5 legend.

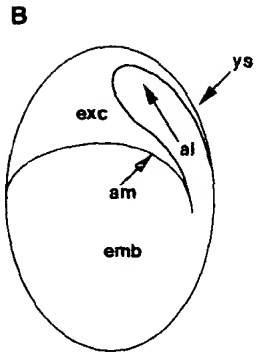
sion between the allantois and amnion does not appear to be enduring because attachment between donor allantoic tips and the host amnion is a rare event (Downs and Gardner, 1995).

1. Genetic Control of Allantoic Morphology: *Brachyury*

The only mutation known to severely affect the morphogenesis of the allantois is *Brachyury (T)*. *Brachyury* was recognized about 70 years ago because mice with one copy of this mutation have short, kinked tails (Dobrovolskaia-Zavadskaia, 1927). Further analysis of this mutation revealed that embryos with two copies of *Brachyury (T/T)* died during midgestation (about 10.5 dpc). The major defects were found in the posterior region of the embryos, notably in the primitive streak, the notochord, and the allantois. In homozygotes, the allantois fails to grow substantially into the exocoelomic cavity and fuse with the chorion (Gluecksohn-Schoenheimer, 1944; Fig. 7).

The allantoic defect of *Brachyury* has not been studied, but it appears that it may arise from the abnormal deployment of allantoic mesodermal cells emerging from the primitive streak (Beddington *et al.*, 1992). The situation may be more complex, however, because fate mapping has demonstrated that the yolk sac and chorionic mesoderms are deployed from the same proximal epiblast tissue as the allantois (Lawson *et al.*, 1991), and yet these tissues do not appear to be affected in *Brachyury*, at least at the gross morphological level.

Several new lines of evidence have provided clues regarding the allantoic defect in *Brachyury* mutants and the apparent escape of the defect by the yolk sac



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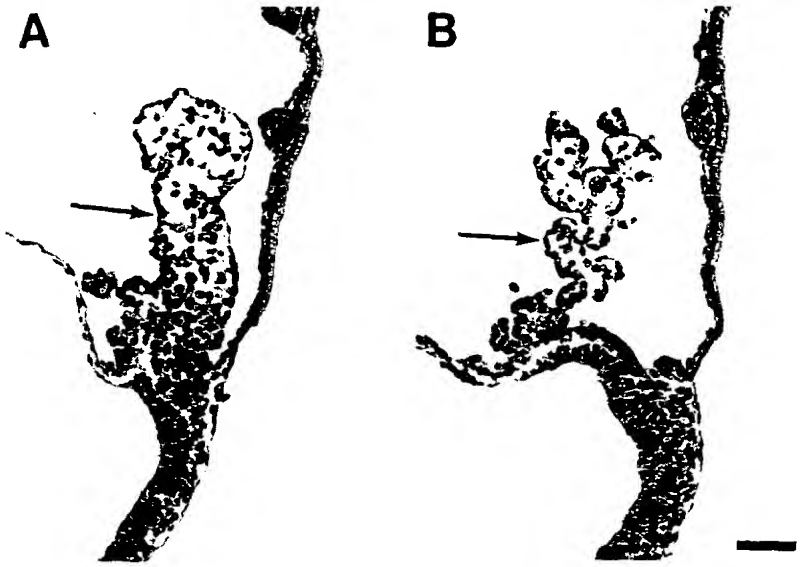

Fig. 7 Comparison of wild-type and putative *Brachyury* allantoises. Matings between heterozygous *T/+* adult mice produced a litter in which the number of embryos displaying normal (A) and defective (B) allantoises (arrows) appeared in the expected ratio. The arrow points to the allantois. Please see Fig. 1 for other morphological features. Scale bar: 53 μm (A); 50 μm (B). Methods: Conceptuses from a single litter of a *T/+* intercross were removed from their implantation sites at nominal day 8.0 postcoitum, and Reichert's membrane was reflected. The conceptuses were then fixed in Bouin's solution for 24 hr, embedded in paraffin, sectioned at 7 μm, dewaxed, and stained in hematoxylin and eosin.

and chorionic mesoderms (Clements *et al.*, 1996). Transgenic mice expressing the *lacZ* reporter gene under the control of a *Brachyury* promoter were used to recapitulate normal *Brachyury* expression in the primitive streak during gastrulation. This sensitive method revealed that *Brachyury* was expressed in the base of the future allantois at the earliest stages that extraembryonic mesoderm forms, i.e., during the mid-to-late streak stage. Previously, this expression had gone unreported (Herrmann, 1991), but careful scrutiny of the expression patterns shown in Figures 1A and 1E of Herrmann (1991) clearly reveal that the base of the allantois expresses *T* as early as the late streak stage.

When a small deletion was included in the promoter of the *lacZ*-tagged *Brachyury* gene and a transgenic mouse line was made from this construct, a somewhat surprising change in the expression pattern of *Brachyury* was observed in gastrulating embryos: the earliest extraembryonic mesoderm to arise from the posterior streak expressed lacZ at the midstreak stage, but later emerging mesodermal cells did not. In particular, expression of the transgene was conspicuously absent from the base of the allantois.

These observations fit in very well with the author's hypothesis that formation of the components of the exocoelomic cavity occurs in three waves and may explain why the yolk sac and chorionic mesoderm may not be affected in *Brachyury* mutants but the allantois is. They suggest that the Brachyury protein product, although expressed, may not be required for the first wave of formation of extraembryonic mesoderm. However, it does appear to be necessary for continued proliferation of extraembryonic mesoderm shortly thereafter (Clements *et al.*, 1996). This would coincide with the second wave of formation of extraembryonic mesoderm, which goes into the amnion and allantois and may explain why the allantois and possibly the amnion are morphologically and functionally defective in *Brachyury* mutants (Rashbass *et al.*, 1991).

The Brachyury gene product appears to be a DNA-binding protein (Kispert *et al.*, 1995). The downstream genes regulated by *Brachyury* have not been identified, but they may be part of the family of adhesion molecules. This is because the cell surface appears to be altered in *Brachyury*, as *T/T* mutant cells form smaller aggregates than their normal counterparts (Yanagisawa and Fujimoto, 1977). Ultrastructural examination of the embryonic portion of the conceptus, and not the allantois, has also revealed that the extracellular matrix in *T/T* embryos is greatly decreased (Jacobs-Cohen *et al.*, 1983). Chimeric studies in which the deployment of *T/T* mutant ES cells was examined revealed a buildup of *T/T* mutant cells in the posterior region and overall reduced motility of these cells, in accordance with the theory that the surface of *Brachyury* mutant cells is altered (Rashbass *et al.*, 1991; Beddington *et al.*, 1992). *Brachyury* allantoises form, but they are misshapen and adhere to the amnion, suggesting that *Brachyury* allantoises have altered surface properties the allow them to adhere to the amnion or that the amnion is affected as well.

C. The Base of the Allantois and Primordial Germ Cells

It is well-established that the germ line is formed from the epiblast of the mouse gastrula (Gardner and Rossant, 1979; Gardner *et al.*, 1985) and that the precursors of the germ line, called the primordial germ cells (PGCs), are found in the hindgut and then migrate along the dorsal mesentery to colonize the gonads by about 11.5 dpc (Mintz and Russell, 1957; Gomperts *et al.*, 1994). Alkaline phosphatase (AP) activity has long been considered to be diagnostic of primordial germ cells because, during migration to the gonads, a large number of cells along the dorsal mesentery express alkaline phosphatase activity that is maintained in the gonads (Mintz and Russell, 1957). The most convincing evidence that these alkaline-phosphatase-positive cells are the PGCs derives from a comparison between wild-type embryos and embryos mutated in the genes for dominant white spotting (*W*) and steel (*Sl*). The mutant embryos revealed a severe deficiency in AP^+ cells along the dorsal mesentery and in the gonads (Mintz and

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Russell, 1957). Further convincing support for AP reactivity as diagnostic for migrating PGCs was obtained from the demonstration that the AP^+/SSEA-1 cells emigrating from the hindgut become tethered to each other via cytoplasmic processes along the dorsal mesentery, allowing them to link up to form exclusive networks that culminate in their aggregation in the gonads (Gomperts *et al.*, 1994).

The location of the primordial germ cells before migration is less clear. Alkaline-phosphatase-positive cells have been traced back from the dorsal mesentery to the yolk sac of the conceptus at about 8.5 dpc (Chiquoine, 1954) and to the base of the allantois, which is particularly rich in AP^+ cells, at about 8.0 dpc (Oździenski, 1967). Oździenski (1967) proposed two hypotheses to explain the significance of AP^+ cells in the base of the allantois. Either they are the PGCs, having been set aside there and eventually returning to the fetus to populate the gonads, or they will contribute only to somatic lineages. The hypothesis that AP^+ cells in the base of the allantois are the primordial germ cells is complicated by the observation that many cells and tissue types, including the epiblast, exhibit alkaline phosphatase activity during gastrulation (MacGregor *et al.*, 1995). Further, because tissue-nonspecific alkaline phosphatase, expressed in migrating PGCs, is not required for formation of the germ line (MacGregor *et al.*, 1995), it is impossible to identify histologically which population of AP^+ cells, if any, are the PGCs.

The only method for resolving whether the cells in the base of the allantois are the PGCs is to fate map this region. If cells residing in the base of the allantois return to the fetus and colonize the gonads, then it is likely that the base of the allantois contains the precursors of the mammalian germ line. It would also be useful to discover the mechanism of expansion of the allantois to demonstrate whether it is at all feasible that a small cluster of AP^+ cells can remain fixed in the base of the allantois from the midstreak to the early somite stage, approximately 42 hr, as previously suggested (Ginsberg *et al.*, 1990).

Preliminary steps were taken to discover the fate of cells in the base of the allantois (Downs and Harmann, 1997). Orthotopic transplantation revealed that cells in the base do not return to the fetus (Downs and Harmann, 1997). Rather, although relatively pluripotent, they appear to contribute only to the allantois and move distally during allantoic growth, contributing to the mature allantois.

However, the theory that the base of the allantois nevertheless contains the future germ line cannot be ruled out on several grounds. The first is that there is no precise morphological feature that clearly defines where the base of the allantois begins. This was apparently the reason that Copp *et al.* (1986) included the entire allantoic bud in orthotopic transplantations designed to elucidate the location of the primordial germ cells in gastrulation. There is also the possibility that the germ cells failed to survive transplantation. This seems unlikely, however, as previous studies have shown that cells taken from this region survive culture and extensively contribute to somatic chimeras (Matsui *et al.*, 1992). In

one instance, these cultured cells even contributed to the germ line (Labosky *et al.*, 1994). Finally, although the base of the allantois had been defined according to Oździenski (Fig. 3) in the transplantation studies, it had previously been demonstrated that allantoic tissue can be regenerated following aspiration of the headfold-stage allantois (Downs and Gardner, 1995). This argues that removal of the allantois via aspiration is either incomplete or, if it is complete, cells beneath the allantois retain the potential to regenerate an allantois (Downs and Gardner, 1995). Therefore, any future studies designed to analyze fate in the base of the allantois will need to precisely define the allantoic–posterior primitive streak region.

VI. Function of the Allantois

A. Vasculogenesis in the Allantois

Formation of large embryonic blood vessels and the vitelline vasculature is thought to occur by a uniquely embryonic process called vasculogenesis [reviewed in Risau and Flamme (1995)]. Pluripotent cells differentiate *in situ* into angioblasts, which subsequently coalesce to form a rudimentary endothelial scaffolding that will be remodeled until the mature blood vessels are formed. The other type of vessel formation, angiogenesis, occurs by sprouting of extant endothelium and occurs throughout the life of the organism.

How the murine umbilical vasculature forms is not known. Given that vascularization is thought to involve mesodermal–endodermal interactions, it is far from clear how the allantois, which is wholly mesodermal, vascularizes.

Some aspects of allantoic vasculogenesis have come to light. In particular, the fate and developmental potency of allantoic mesoderm at the headfold stage have been analyzed in a series of transplantation experiments (Downs and Harmann, 1997). Results of these studies demonstrated that angioblasts are present in the allantois as early as the headfold (presomite) stage. The headfold stage was chosen because it is the earliest time at which the allantois could be subdivided into thirds along its proximodistal axis (Figs. 3 and 8). The tip was thought to be specialized for chorioallantoic fusion, and the base was thought to protect or induce the future germ line (Section IV.C). On the basis of these suppositions, it was envisioned that the allantois may exhibit distinct cellular properties along its proximodistal axis.

lacZ-expressing allantoises were subdivided into three regions, the base, midportion, and tip, and clumps of cells from each region were placed into three sites in the conceptus (Fig. 8). When placed into the fetus, none of the allantoic regions colonized paraxial (somitic) mesoderm, but all three regions colonized the endothelium of the dorsal aorta and adjacent mesenchyme. The allantoic midportion colonized the dorsal aorta most avidly. Only the base exhibited a

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ich the allantois could be subdivided
s. 3 and 8). The tip was thought to be
the base was thought to protect or
On the basis of these suppositions, it
it distinct cellular properties along its

ded into three regions, the base, mid-
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nto the fetus, none of the allantoic
lerm, but all three regions colonized
djacent mesenchyme. The allantoic
t avidly. Only the base exhibited a

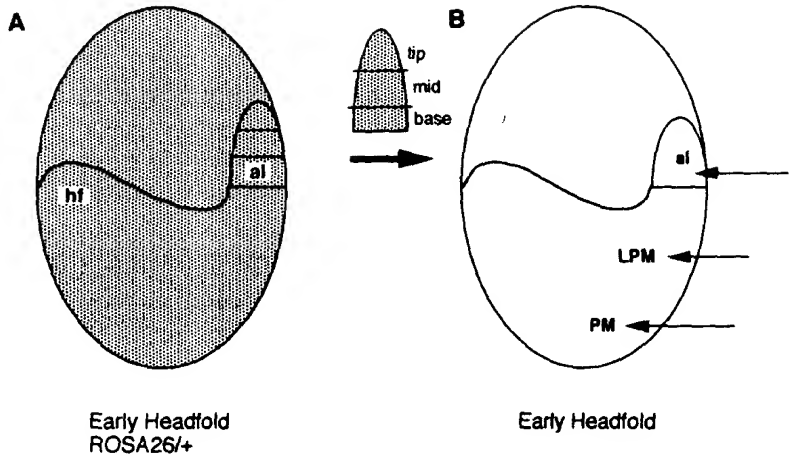


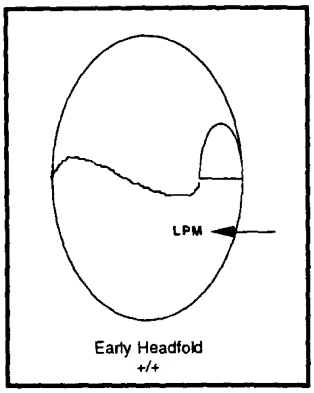
Fig. 8 Sites of transplantation. Donor allantoises from headfold-stage ROSA26* *lacZ*/+ hemizygous donor conceptuses (A) (Downs and Harmann, 1997) were removed and subdivided into three regions: tip, middle portion (mid), and base (see also Fig. 3). Clumps from each allantoic region were transplanted into each of three headfold-stage host sites in a nontransgenic host (B): the base of the allantois (al), the primitive streak at the level of prospective lateral plate mesoderm (LPM), and the primitive streak at the level of prospective paraxial mesoderm (PM). Other abbreviations: hf, region of the headfolds.

tendency to colonize a few more cell types, including lateral plate mesoderm, surface ectoderm, and the endoderm of the future coelom. These data are summarized in Figs. 9 and 10.

Transplantation of all three allantoic regions back into the allantois revealed that a gradient of differentiation may exist along the proximodistal axis of the headfold-stage allantois (Fig. 11). This was reflected in the observation that, despite being placed into the base of the hose allantois, all three donor allantoic regions displayed colonization patterns of the host allantois that were distinctly different from each other. Donor basal cells typically moved only as far as the midregion of the host allantois, whereas tip cells most often returned to the tip (Fig. 12).

These studies suggested that the allantois undergoes vasculogenesis rather than angiogenesis because allantoic angioblasts solely contributed to large vessel endothelium. Preliminary transplantation experiments of the yolk sac have tentatively confirmed this hypothesis, revealing that there is no contribution of the yolk sac to the allantois until at least the 10 somite stage (K. Downs, S. Gifford, and M. Blahnik, unpublished data). Thus, the allantois may be the exception to the rule that angioblast formation is dependent upon mesodermal interactions with endoderm.

If angioblasts are formed *de novo* within the allantois, then how do the extraembryonic mesodermal cells that comprise the allantoic bud differentiate into



75.0 - 99.9%
50.0 - 74.9%
10.0 - 49.9%
1.0 - 9.9%
\+ <1.0%

Summary of Grafts into the Primitive Streak at the Level of Prospective Lateral Plate Mesoderm

	Orthotopic: Prospective Lateral Plate Mesoderm	Heterotopic: Allantois to Prospective Lateral Plate Mesoderm		
Tissue Grafted→ Tissue Colonized ↓	LPM	Base	Mid-Portion	Tip
Lateral Plate Mesoderm		+		
Intermediate Mesoderm				
Somites				
Neural Tube				
Capillaries				
Endoderm of Future Coelom				
Surface Ectoderm		+		
Endothelium of Intersegmental Vessels				
Endothelium of Aorta				
Mesenchyme Adj to Dorsal Aorta				
Umbilical Endothelium at All/Am Junction		+		
No. Chimeras with Unincorporated Donor Cells	0	0	5	2
Initial Stage of Host Embryo	Headfold, 2-3	Headfold	Headfold, 3.4	Headfold
Final No. Somite Pairs	11-15	10-12	10-16	11-16
Total Number of Incorporated Transgenic Cells	1925	2789	2769	995
Number Chimeras (% of Total Injected)	10 (52.6%)	4 (23.5%)	12 (54.4%)	5 (27.8%)

angioblasts? One model that can be evoked is dependent upon spatial cues. For example, extraembryonic mesoderm destined for the allantois emerges from the primitive streak in a relatively pluripotent state, but as it is pushed distally by sustained addition of nascent extraembryonic mesoderm into the allantoic base, it moves out of the sphere of influence of the primitive streak and differentiates into angioblasts. Movement farther into the tip entails further differentiation, into either chorioadhesive cells or possibly specialized umbilical endothelial cells.

The second model is a temporal one. The fate of allantoic cells may be dependent upon the amount of time they reside in different allantoic levels, the length of which may in turn be dependent upon the rate at which cells are recruited into the base of the allantois and/or the number of cell cycles completed by allantoic cells before distal translocation.

B. Erythropoietic Potential of the Allantois

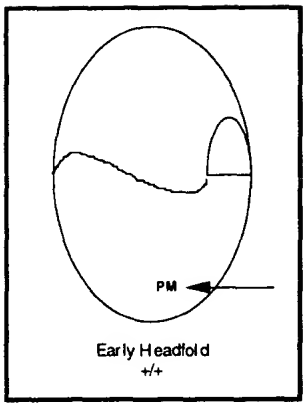
Although red blood cells are obvious in the allantois shortly after allantoic vasculogenesis begins, it has been assumed that these come from either the yolk sac or the fetus. Bloom and Bartelmez (1940) reported profiles of hematopoietic cells in the nascent human allantois, but concluded on the basis of histological evidence that these were the result of "abortive" blood cell formation in belly stalk (umbilical) vessels. Gene expression studies that localize erythropoietic transcription factors to component tissues of the exocoelom during gastrulation have offered few clues as to whether the allantois may be an erythropoietic organ, for focus is typically on the yolk sac.

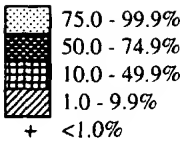
It was reported that allantoises cultured in isolation do contain cells that stain positively for benzidine, which is indicative of the presence of hemoglobin (Downs and Harmann, 1997). However, because the allantoises had been removed through the yolk sac, contamination by yolk sac blood islands or induction of allantoic hemoglobin synthesis by factors emanating from the broken yolk sac could not be ruled out. If the latter is correct, the allantois may possess the developmental potential to become erythropoietic under appropriate conditions and could, at the very least, turn out to be an attractive system for studying the genetic control of erythropoiesis.

75.0 - 99.9%
50.0 - 74.9%
10.0 - 49.9%
1.0 - 9.9%
<1.0%

nitive Streak at the Level of Plate Mesoderm		
Heterotopic: Allantois to rospective Lateral Plate Mesoderm		
Base	Mid-Portion	Tip
+		
+		
+		
0	5	2
adfold	Headfold, 3,4	Headfold
0-12	10-16	11-16
789	2769	995
4 3.5%)	12 (54.4%)	5 (27.8%)

Fig. 9 Summary of allantoic transplantation into the prospective primitive streak at the level of lateral plate mesoderm. Control orthotopic and experimental heterotopic grafts of allantoic tissue into the prospective lateral plate mesoderm are summarized. The total number of grafted cells for each series is reported at the bottom of the figure. The filled-in boxes represent the percentage of grafted cells found in each of the host's tissues described in the leftmost portion of the figure. Original data are in Downs and Harmann (1997).





Summary of Transplants into the Primitive Streak at the Level of Prospective Paraxial Mesoderm

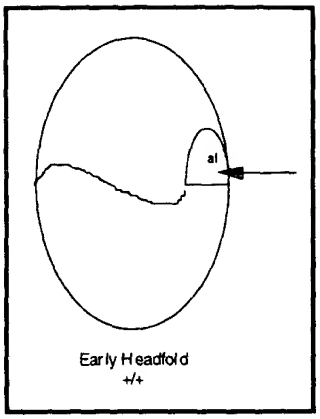
	Orthotopic: Prospective Paraxial Mesoderm	Heterotopic: Allantois to Prospective Paraxial Mesoderm		
Tissue Grafted→ Tissue Colonized ↓	PM	Base	Mid-Portion	Tip
Somites				
Pre-Somitic Mesoderm				
Capillaries (in neurectoderm)				
Neural Tube				
Notochordal Plate				
Surface Ectoderm	+			
Endothelium of Intersegmental Vessels				
Endothelium of Dorsal Aorta				
Mesenchyme Adj to Dorsal Aorta				
No. Chimeras with Unincorporated Donor Cells	1	0	4	1
Initial Stage of Host Embryos	Headfold, 3-4	Headfold	Headfold, 1	Headfold
Final Number Somite Pairs	10-14	9-11	8-13	11-13
Total Number of Incorporated Transgenic Cells	1021	136	1060	340
Number Chimeras (% Total Injected)	7 (33.3%)	2 (10.5%)	6 (28.6%)	4 (30.8%)

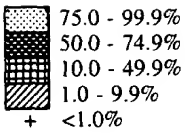
75.0 - 99.9%
50.0 - 74.9%
10.0 - 49.9%
1.0 - 9.9%
<1.0%

mitive Streak at the Level of Mesoderm		
	Heterotopic: Allantois to rospective Paraxial Mesoderm	
se	Mid-Portion	Tip
)	4	1
fold	Headfold, 1	Headfold
11	8-13	11-13
6	1060	340
5%)	6 (28.6%)	4 (30.8%)

Fig. 11 Gradient of differentiation in the allantois. Schematic diagram depicting the topographical arrangement of pluripotent mesodermal cells and angioblasts in the headfold-stage allantois (Downs and Harmann, 1997). The allantois is continuous with the primitive streak (PS). Cells from the epiblast ingress into the primitive streak where they are transformed into pluripotent extraembryonic mesodermal cells. These pluripotent cells move distally into the allantoic base (indicated by the arrow extending from the PS to the distal allantoic region). As more extraembryonic mesodermal cells are added from the streak to the allantois, the basal cells move distally, first into the midregion where they become differentiated into angioblasts and second into the distal region, where some cells may continue to differentiate into angioblasts and others into cells specialized for fusion with the chorion or endothelium at the chorioallantoic fusion junction.

Fig. 10 Summary of allantoic transplantation into the prospective primitive streak at the level of paraxial mesoderm. Control orthotopic and experimental heterotopic grafts of allantoic tissue into the prospective paraxial mesoderm are summarized. The total number of grafted cells for each series is reported at the bottom of the figure. The filled-in boxes represent the percentage of grafted cells that was found in each of the host's tissues described in the leftmost portion of the figure. Original data are in Downs and Harmann (1997).





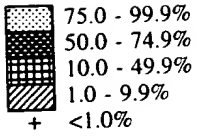
Summary of Grafts into the Base of the Allantois			
Region Grafted→ Region Colonized ↓	**Base**	**Mid-Portion**	**Tip**
Base	+	+	0
Mid-Region			
Distal Third			
Vitelline Omphalomes Artery	+	+	
No. Chimeras with Unincorporated Donor Cells	1	2	0
Initial Stage of Host Embryos	Headfold	Headfold, 3-5	Headfold, 3
Final Number Somite Pairs	10-16	8-16	11-16
Total Number of Incorporated Transgenic Cells	5652	9715	2501
Number Chimeras (% Total Injected)	21 (60.0%)	16 (76.2%)	12 (85.7%)

Fig. 12 Summary of allantoic transplantation into the base of the allantois. The key to this figure is similar to that for Figs. 9 and 10, with the exception that there is one less column, as the control orthotopic grafts were of the base of the allantois. Original data are in Downs and Harmann (1997).

VII. The Allantois in Fetal Therapy

The principal function of the allantois is to form the vascular connection between mother and fetus. The umbilical vessels provide a direct gateway to fetal circula-

Karen M. Downs



Base of the Allantois	
Mid-Portion	**Tip**
+	0
+	
2	0
Headfold, 3-5	Headfold, 3
8-16	11-16
9715	2501
16 (76.2%)	12 (85.7%)

e base of the allantois. The key to this figure is
n that there is one less column, as the control
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form the vascular connection between
vide a direct gateway to fetal circula-

tion and a potentially invaluable system for the continuous delivery of blood-borne therapeutic factors to the fetus during gestation. Data have been presented in this chapter that demonstrate that, with little effort, the murine allantois can be manipulated extensively without compromising the development of the fetus These results, along with others that have demonstrated gene expression from endothelial cell-specific promoters (Korhonen *et al.*, 1995; Schlaeger *et al.*, 1995), suggest that the umbilical cord has the potential to express therapeutic factors via the endothelial cells that comprise its blood vessels, with subsequent delivery of the factor to fetal circulation. The mouse may be an ideal system for testing the soundness of umbilical therapy.

The success of fetal therapy via the umbilical cord rests upon obtaining allantoic cells, either primary cells or allantoic cell lines whose properties are similar enough to intact allantoic cells that they can colonize the umbilical cord. Because the location of pluripotent allantoic cells and angioblasts is now more clear (Downs and Harmann, 1997), these cells may be exploited for umbilical therapy These cells or cell lines derived from them may be reintroduced into the umbilical cords of developmentally compromised fetuses as wild-type cells in cases where the therapeutic factor of interest is produced normally in endothelial cells and in sufficient quantity to rescue the fetal defect via secretion of the factor into the fetal bloodstream. Alternatively, where the gene is not expressed normally in endothelial cells or where large amounts of the gene product are needed, allantoic cells may be transfected with a therapeutic gene of interest under the regulation of a robust endothelial cell-specific promoter. Such genetically engineered cells would then be placed into the umbilical cord, assimilate, differentiate into endothelial cells, and express the gene of interest, secreting it into the circulation (Fig 13).

Umbilical therapy may be critical in cases where the therapeutic factor may be toxic to the mother, cannot cross the placental barrier, or whose half-life is so short as to make intermittent injection into the umbilical cord impractical and costly. Delivery of recombinant factors in fetal life, while the immune system is developing, may permit some therapeutic factors to be tolerated by the immune system later in life. One potentially attractive benefit to umbilical therapy is that because the placenta will be shed at birth, there will be no unforeseen deleterious effects of this approach on the adult.

Key to the success of this therapy are considerations of fetal diagnosis early in pregnancy, the source of the umbilical cells, and the possibility of tissue rejection, as well as the number of cells required for successful umbilical chimerism None of these parameters have been considered here.

An example of a disease for which umbilical therapy may be beneficial is hemophilia A. Hemophilia A affects 1 in 5,000–10,000 males in all populations and is caused by a defect in clotting Factor VIII. What makes hemophilia A particularly difficult to treat is that many patients will develop inhibitors against recombinant Factor VIII (Bi *et al.*, 1995). Therefore, a possible means for pre-

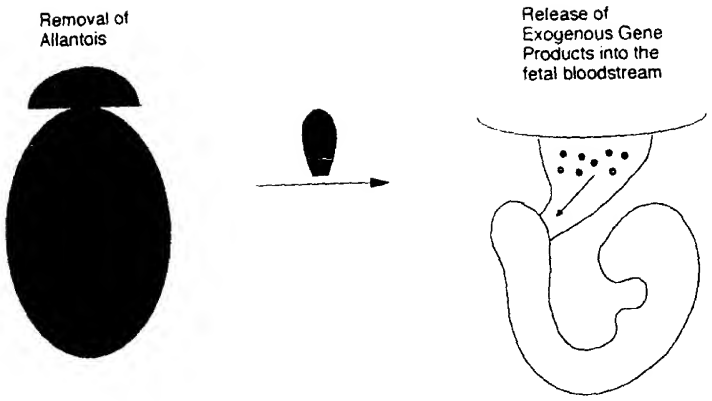
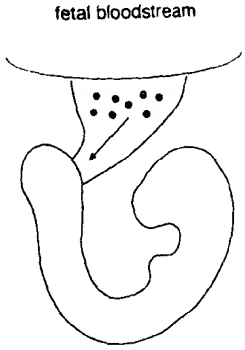


Fig. 13 Umbilical gene therapy. Allantoic cells are removed from an appropriately staged conceptus and introduced into the allantois or umbilicus of a developmentally compromised fetus. There they assimilate into the umbilicus and differentiate into endothelial cells that can deliver either sufficient amounts of wild-type therapeutic factor to the fetal bloodstream or high levels of the therapeutic factor if the allantoic cells have been genetically altered to overproduce the factor. At birth, the chimeric umbilical cord will be shed.

venting the formation of these inhibiting antibodies would be to begin therapy *in utero* as the immune system is developing. This would ensure that Factor VIII is not recognized as a foreign protein when given after birth. However, because Factor VIII does not cross the placental barrier (Lozier and Brinkhous, 1994) and the half-life of Factor VIII is only 10 hr (Brownlee, 1995), delivery of Factor VIII to the fetus via the mother or by injection into the umbilical cord is impossible or impractical.

Factor VIII is produced in the liver, although its exact site of production is still controversial (Sadler and Davie, 1994). Factor VIII mRNA has been detected in extrahepatic tissues such as the spleen, lymph nodes, and kidneys (Brownlee, 1995). In the liver, Factor VIII is processed in the endoplasmic reticulum and Golgi, where proteolytic cleavage produces a heavy and a light chain held together by metal ions. The Factor VIII heterodimer is then secreted as a glycoprotein into the blood, where it circulates as a complex with von Willebrand factor, itself a multimer. Although Factor VIII requires the presence of von Willebrand factor for stability (Brownlee, 1995), previous studies have revealed that the endothelial cells of the umbilical cord are the principal producing cells of von Willebrand factor (Dichek and Quertermous, 1989). Therefore, Factor VIII should be stable in the system proposed here, as the chimeric umbilical cord will express both Factor VIII and von Willebrand factor.



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VIII. Conclusions

The allantois, heretofore little studied, may provide fresh approaches to old problems. Because the principal function of the allantois is to vascularize, the allantois is a very attractive organ for the study of vasculogenesis, especially as it can be isolated free of contamination from embryonic and extraembryonic tissues. The mechanism by which differentiation along the proximodistal axis of the allantois is established may shed new light on how morphogenetic gradients are set up in the mammal. If the allantois possesses erythropoietic potential, then it may be an ideal system for studying the genetic control of erythropoiesis. Ultimately it is hoped that the results of basic research combined with the privileged position of the allantois in fetal development will stimulate advances in methods of fetal therapy.

Acknowledgments

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